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Activation of human B lymphocytes by nanogram concentrations of anti-IgM-dextran conjugates*

Surface immunoglobulin (sIg) cross-linking on B lymphocytes by high concentrations of anti-Ig antibody has been used to mimic antigen-stimulated B cell activation. In order to develop a system to study sIg-mediated Tcell-independent B cell activation using low concentrations of anti-Ig antibody that more closely resemble the concentrations of antigen that are achieved under *in vivo* conditions, we conjugated monoclonal anti-human IgM antibody (anti-μ) to dextran (molecular weight 2×10^6) thereby increasing its valency. This dextran conjugate (anti-μ-Dex) stimulated comparable levels of thymidine incorporation and B cell size increases as were seen with unconjugated anti-μ but at 100- to 1000-fold lower concentrations. Anti-μ-Dex also stimulated increases in intracellular ionized calcium ($[Ca^{2+}]_i$) in a higher percentage of cells, of greater magnitude and of longer duration than that stimulated by unconjugated anti-μ. Interestingly, there was no direct correlation between the increases in $[Ca^{2+}]_i$ that were stimulated by anti-μ-Dex and its ability to stimulate B cell proliferation. The concentrations of anti-μ-Dex (10 μg/ml) that led to the highest increase in $[Ca^{2+}]_i$ resulted in thymidine incorporation that was no greater than that of medium control, whereas 0.01 to 0.1 μg/ml stimulated significant thymidine incorporation with 50% lower levels of stimulation of $[Ca^{2+}]_i$. These data demonstrate that anti-μ-Dex is a potent activator of human B lymphocytes, is effective even at ng/ml concentrations which over a 2-h time period do not induce detectable modulation of sIg, and its stimulation of B cells into G_1 and S may not be directly related to its ability to stimulate increases in levels of $[Ca^{2+}]_i$.

1 Introduction

Cross-linking of sIg on B lymphocytes by anti-Ig antibodies has been used as a model to study B cell activation that is stimulated by cross-linking of sIg by antigen [1–4]. The ability to cross-link sIg is central to the generation of activation signals [4–9]. Thus, Fab fragments which do not cross-link sIg do not induce B cell proliferative responses as do $F(ab')_2$ fragments of antibody; likewise, univalent antigens are weak immunogens as compared to multivalent antigens [10]. Increasing the valency of anti-Ig antibody or of antigen by insolubilization onto Sepharose beads renders them much more potent activators of B cells [1, 11–13]. While in many respects these multivalent conjugates provide good model systems to study B cell activation, the fact that they are particulate and insoluble diminishes their utility as models for studying activation requirements stimulated by soluble antigens. The primary limitation to using soluble anti-Ig antibody as a model for soluble antigen is that high molar concentrations of anti-Ig antibody that may not be achieved by physiologic antigens *in vivo* are required and at these high concentrations they mediate the rapid modulation of sIg. Recently, a model has been described which utilizes anti-Ig conjugated to high-molecular weight dextran (anti-Ig-Dex) to stimulate murine B cell proliferation [14]. Anti-Ig-Dex induced higher levels of murine B cell proliferation and increases in expression of MHC class II molecules on B cells than did unconjugated anti-Ig at concentrations which were 1000-fold lower. At these low concentrations, which more closely resemble the concentrations of antigens that are achieved *in vivo*, sIg is not modulated from the surface of the murine B cell. This system may provide a model for studying Tcell-independent B cell activation that is mediated by sIg cross-linking without the requirement for purifying Ag-specific B cells.

The present studies were undertaken to determine whether anti-Ig-Dex conjugates can be used to study the activational events of purified human B cells. We demonstrate here that anti-μ-Dex is 100- to 1000-fold more potent than unconjugated anti-μ in inducing increases in human B cell size, elevations of intracellular calcium ($[Ca^{2+}]_i$), and enhancement of proliferation. Furthermore, while unconjugated anti-μ induces B cell activation only at concentrations which modulate sIg, Dex-conjugated anti-Ig mediates B cell activation even at non-modulatory concentrations of antibody.

[I 8303]

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Abbreviations: **Anti-μ:** Mouse anti-human IgM mAb **Anti-μ-Dex:** Mouse anti-human IgM mAb conjugated to high molecular weight dextran **SAC:** *S. aureus* Cowan A I **$[Ca^{2+}]_i$:** Intracellular Ca^{2+} concentration

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2 Materials and methods

2.1 Antibodies

FITC-labeled affinity purified $F(ab')_2$ goat anti-human IgM (GaHμ) antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Mouse anti-human IgM mAb (anti-μ) antibodies (clone DA4-4) were obtained from American Type Culture Collection (ATCC; Rockville, MD). Murine mAb 9.6 (anti-CD2), G10-1 (anti-CD8), and FC-2 (anti-CD16) were a generous gift from Dr. Jeffrey Ledbetter (Oncogen Corp., Seattle, WA). Antibodies 20.3 (anti-CD14), and 38.1 (anti-CD3) were a generous gift from Dr. John Hansen (Fred Hutchinson Cancer Research Center, Seattle, WA). Anti-CD11 (OKM1) antibody was isolated from cells obtained from ATCC. Murine anti-human NKH-1 and FITC-conjugated anti-human B-1 (anti-CD20)-specific mAb were obtained from Coulter Clone (Hialeah, FL). Anti-human leukocyte (HLe-1, anti-CD45) antibody was obtained from Becton Dickinson (Mountain View, CA). FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse Ig (GaMIg) were obtained from Tago (Burlingame, CA).

2.2 Preparation of antibody-Dex conjugates

Conjugation of mouse anti-human IgM (anti-μ) mAb to high molecular weight Dex was performed as described previously [14]. Briefly, anti-μ antibodies were covalently linked through stable thioether bonds to molecules of aminoethylcarbamylmethyl-dextran using a heteroligation technique. The antibody was lightly S-acetylthiolated and the Dex was acylated by introduction of iodoacetamido groups. The two components were then mixed in the presence of hydroxylamine which removed the S-acetyl group. The exposed free sulfhydryl reacts *in situ* with the iodoacetamido groups.

2.3 Human B cell growth factor and *S. aureus* Cowan A I

Low molecular weight BCGF was obtained from Cellular Products, Inc. (Buffalo, NY). *S. aureus* Cowan A I (SAC) was purchased from Calbiochem-Behring (La Jolla, CA).

2.4 B cell preparation

Mononuclear cells were obtained by peripheral blood leukopheresis of healthy adult blood donors (National Institutes of Health Blood Bank). Separation of these PBMC on the basis of size and density was achieved by counterflow centrifugal elutriation as described [15, 16], yielding a uniform monocyte-free B cell-enriched population (fraction 2) of small dense lymphocytes. These cells were subsequently treated with saturating amounts of anti-human mAb specific for CD2, CD3, CD8, CD11, CD14, CD16 and NKH-1 to coat non-B cells at 4°C for 50 min. This was followed by treatment with goat anti-mouse IgG-coated magnetic beads (Dynabeads-M450, Dynal, Inc., Oslo, Norway) using three beads/cell at 4°C for 60 min [17, 18]. The cells obtained by this method contained < 0.1% monocytes by esterase staining, and were at least 95% B1 (CD20) positive by FCM analysis.

2.5 Cell culture and proliferation

Cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) medium that contained L-glutamine, gentamycin (50 μg/ml), and 10% LPS-free FCS (Hyclone, Logan, UT) in flat-bottom microtiter plates (Nunc, Roskilde, Denmark) at densities of 1×10^5 in 0.2 ml medium/well. Cultures were incubated in a humidified 5% CO_2 incubator at 37°C for 72 h. The cultures were pulsed 18 h before harvesting with 1.0 μCi = 37 kBq of [^{3}H]dThd. The time of harvest was recorded as the time of assay. Each culture was performed in triplicate with results expressed as the arithmetic mean ± SD of cpm/culture.

2.6 Analysis of cell size

Cell size was measured with a Coulter Counter and Coulter Channelizer (Coulter Electronics, Hialeah, FL). Purified peripheral blood human B cells were incubated for 24 h at 37°C with unconjugated anti-μ at 100, 10 and 1 μg/ml, and conjugated anti-μ-Dex at 10, 1, 0.1 and 0.01 μg/ml. Cell size changes were compared to cell size prior to incubation and to cells incubated for 24 h at 37°C in the absence of anti-Ig antibody. The data are displayed as population size distribution curves.

2.7 Modulation of B cell sIgM by anti-μ and anti-μ-Dex

To determine the ability of conjugated anti-μ-Dex and unconjugated anti-μ to bind and modulate sIgM, no antibody (control), anti-μ-Dex at 10, 1 and 0.1 μg/ml or anti-μ antibody at 10 and 1 μg/ml were added to B cells for 120 min at 4°C. Unbound antibody was removed by washing the cells twice at 4°C. Aliquots of cells were suspended in FCM medium containing 0.2% sodium azide at 4°C to prevent sIg modulation and were stained with FITC-conjugated GaMIg antibodies. Other aliquots of cells were incubated at 37°C for 2 h to allow for modulation of sIg, and then were washed in medium containing azide at 4°C to arrest further modulation. These cells were then stained with FITC-conjugated GaMIg or GaHμ antibodies, followed by FCM analysis.

2.8 Indo-1 assay of intracellular calcium

Measurement of $[Ca^{2+}]_i$ was performed by using the ratio of Ca^{2+}-dependent indo-1 fluorescence as previously described [19]. The peak mean $[Ca^{2+}]_i$ increases, the percentage of B cells responding, and the integrated area under the curve of the mean $[Ca^{2+}]_i$ above baseline *vs.* time plot were calculated. The integrated area takes into account the magnitude of the increase in $[Ca^{2+}]_i$, the duration of response and the percentage of cells responding.

3 Results

3.1 Stimulation of human peripheral blood B cell proliferation using ng/ml concentrations of anti-μ-Dex

Relatively high, non-physiological concentrations of anti-μ antibodies are required to induce both human and murine B

cell proliferation. Differentiation is not stimulated under these conditions unless soluble T cell-derived lymphokines are added. In contrast, TI type 2 antigens, *e.g.*, TNP-dextran and TNP-Ficoll stimulate both antigen-specific B cell proliferation and differentiation at significantly lower antigen concentrations [20–25]. The enhanced stimulatory capacity of these multivalent haptenated polysaccharides may reflect their ability to mediate efficient cross-linking of sIg. To determine whether in fact this was the case, we compared the ability of a multivalent monoclonal anti-human μ-Dex conjugate (anti-μ-Dex) to induce human B cell activation to that of bivalent unconjugated anti-human μ (anti-μ) mAb. Anti-μ-Dex at 1.0 μg/ml stimulated B cell proliferation of 78% higher magnitude than unconjugated anti-μ at 100 μg/ml concentration (Fig. 1a). The dose-response relationship of these agonists revealed that anti-μ-Dex was as mitogenic as the unconjugated anti-μ at 100- to 1000-fold lower concentrations (Fig. 1a) and stimulated equivalent levels of proliferation at 0.1 μg/ml as did the unconjugated anti-μ at 100 μg/ml. At the lower concentration of 10^{-4} μg/ml anti-μ-Dex stimulated proliferation, which was equivalent to that stimulated by anti-μ at 1 μg/ml. In the presence of B cell growth factor (BCGF) the Dex-conjugated antibody was effective at 10000-fold lower concentrations than unconjugated anti-μ (Fig. 1b). Thus, at 0.01 μg/ml, anti-μ-Dex stimulated proliferation that was equivalent to that stimulated by 100 μg/ml of unconjugated anti-μ. In control cultures in which Dex was added to anti-μ antibody no enhanced proliferative response was observed above that stimulated by anti-μ only (data not shown).

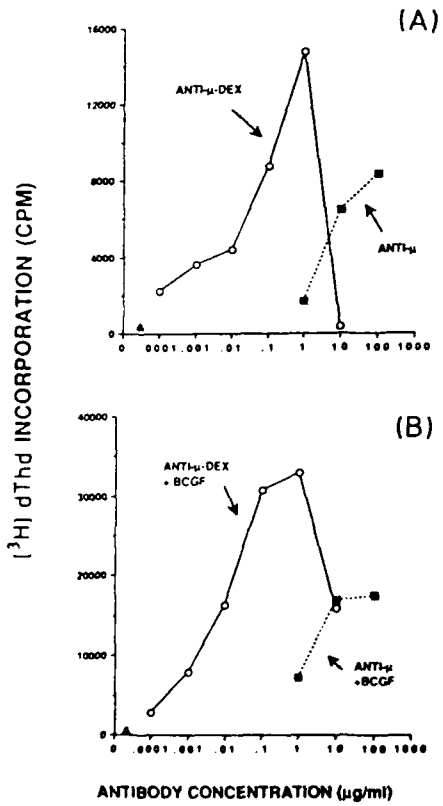

Figure 1. Anti-μ-Dex antibodies stimulate human B cell proliferation at ng/ml concentration. Small, purified human B cells were incubated for 3 days with various concentrations of anti-μ or anti-μ-Dex antibodies in the absence (A) or presence (B) of 10% BCGF. [3H]dThd was added 18 h prior to harvest. Data are presented as arithmetic mean of triplicate cultures.

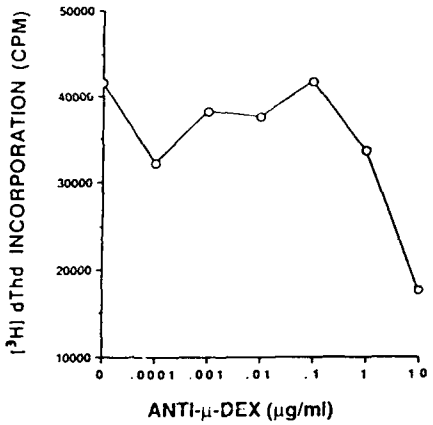

Figure 2. Suppressive effect of high-dose anti-μ-Dex on SAC-induced human B cell proliferation. Purified human B cells were incubated for 3 days with 0.001% SAC in the absence or presence of various concentrations of anti-μ-Dex antibody. [3H]dThd was added 18 h prior to harvest. Data are presented as arithmetic mean of triplicate cultures.

To evaluate whether the absence of proliferation observed when cells were stimulated with anti-μ-Dex at 10.0 μg/ml (see Fig. 1a) represented an inhibitory effect on cell activation or alternatively reflected the lack of a stimulatory effect, we cultured B cells with different concentrations of anti-μ-Dex together with SAC. B cells cultured with SAC only were stimulated to high levels of proliferation, but were significantly inhibited when cultured in the added presence of 10 μg/ml of anti-μ-Dex (Fig. 2). Lower concentrations of anti-μ-Dex did not considerably inhibit SAC-induced proliferation.

3.2 Anti-μ-Dex induces cell size increases at 1000- to 10000-fold lower concentrations and of greater magnitude than that stimulated by anti-μ

The observation that anti-μ stimulated B cell proliferation at 100- to 1000-fold lower concentrations than did unconjugated anti-μ might have reflected either its ability to recruit greater numbers of cells to proliferate or, alternatively, its ability to mediate a more effective activation signal. To study this point we compared the ability of anti-μ and anti-μ-Dex to stimulate increases in B cell size as a reflection of the magnitude of the stimulus delivered at the level of the individual B cells. Purified peripheral blood human B cells were incubated with anti-μ-Dex at concentrations varying from 0.01 μg/ml to 10 μg/ml for 24 h or with unconjugated anti-μ at concentrations of 100, 10 and 1 μg/ml and were sized at 24 h using a Coulter Channelizer. Increases in cell size were observed in B cells that were incubated with anti-μ at 100, 10 and 1 μg/ml, with the lower concentrations of antibody mediating lesser increases in cell size. Increases in cell size were observed at all of the concentrations of the conjugated anti-μ-Dex that we tested.

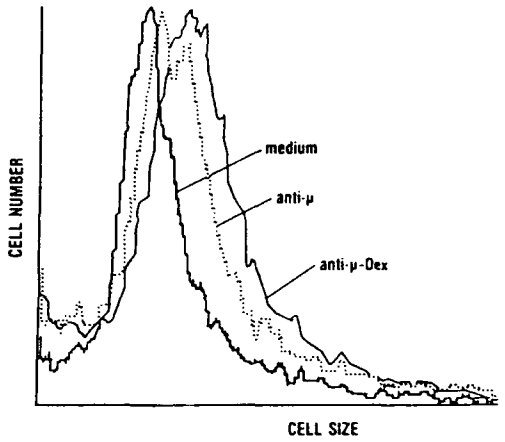

Figure 3. Anti-μ-Dex induces large increases in B cell size. Purified B cells were incubated for 24 h with 100 μg/ml anti-μ, or 0.01 μg/ml of anti-μ-Dex or with medium (control). Cell size was measured by a Coulter Counter and Channelizer.

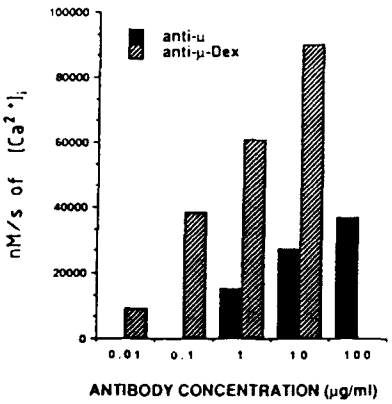

Figure 4. Anti-μ-Dex leads to increases in $[Ca^{2+}]_i$ in human B cells. Purified human B cells were loaded with indo-1 as described in Sect. 2.8. Cells were then stimulated either with anti-μ or anti-μ-Dex and the net cytoplasmic Ca^{2+} mobilization above base line was measured for a period of 100 s. The data are expressed as the integrated area under the response curve.

with no decrement in the magnitude of cell size increase even with decreasing concentrations of anti-μ-Dex. The lowest concentration of anti-μ-Dex, 0.01 μg/ml, stimulated a greater cell size increase than did the unconjugated antibody at 100 μg/ml (Fig. 3). The major shift in size distribution of cells stimulated by anti-Ig-Dex suggest that the majority of the B cells were being stimulated (at least into G_1) by the anti-Ig-Dex conjugates and the enhanced responses that were observed did not reflect heightened responses of a minority B cell population.

3.3 Anti-μ-Dex conjugates induce heightened increases in $[Ca^{2+}]_i$ than unconjugated anti-μ

Increases in $[Ca^{2+}]_i$ have been identified as an early activation event in anti-Ig-stimulated human and murine B cells [8, 26]. To investigate if the difference in stimulatory ability of anti-μ and anti-μ-Dex was reflected at early stages of activation we compared the increases in $[Ca^{2+}]_i$ that were stimulated by anti-μ-Dex and anti-μ. Anti-μ-Dex stimulated equivalent increases in $[Ca^{2+}]_i$ as anti-μ at 100- to 1000-fold lower concentrations with regard to both the mean peak $[Ca^{2+}]_i$ as well as the total integrated area under the curve which represents the magnitude of the calcium mobilization, the duration of the response, and the number of cells responding (Figs. 4 and 5). In addition, the duration of the response induced by anti-μ-Dex was more prolonged than that induced by anti-μ (Fig. 6). While concentrations

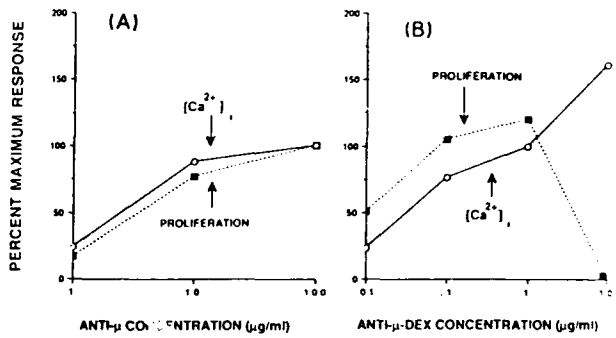

Figure 5. Comparison of increases in peak $[Ca^{2+}]_i$ and [^{3}H] dThd incorporation induced by activation with anti-μ or anti-μ-Dex antibodies. Purified human B cells were loaded with indo-1. Subsequently they were stimulated either with anti-μ (A) or anti-μ-Dex (B) antibodies, and the peak $[Ca^{2+}]_i$ response (open circles) was measured as described in Sect. 2.8. Other aliquots of human B cells from the same donor, obtained on the same day, were incubated with anti-μ or anti-μ-Dex for 3 days. [^{3}H]dThd was added prior to harvest. Data were calculated as arithmetic mean of triplicate cultures (closed squares). Data for both peak $[Ca^{2+}]_i$ and [^{3}H] dThd incorporation by anti-μ or anti-μ-Dex are presented as relative percent, where 100% reflects the response stimulated by 100 μg/ml of anti-μ.

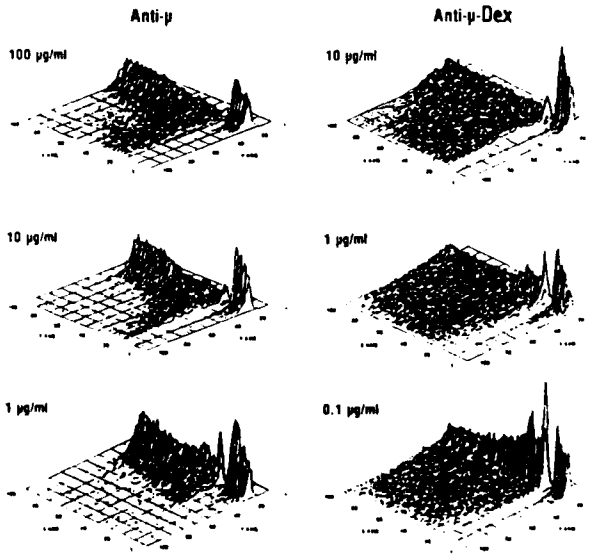

Figure 6. Anti-μ-Dex induces increases in $[Ca^{2+}]_i$ of longer duration than anti-μ. $[Ca^{2+}]_i$ was assayed in purified human B cells by indo-1 fluorescence by FCM analysis after stimulation with the indicated concentration of anti-μ or anti-μ-Dex antibody. Results are displayed in isometric plots of $[Ca^{2+}]_i$ (nM) *vs.* time (min) (x-axis) *vs.* numbers of cells (y-axis).

of 0.1 μg/ml of anti-μ-Dex stimulated large increases in $[Ca^{2+}]_i$ which were similar to those stimulated by 10-fold higher concentrations of unconjugated anti-μ, significantly fewer cells were stimulated by anti-μ-Dex. This is unlike the situation seen with unconjugated anti-μ where both fewer cells and almost negligible $[Ca^{2+}]_i$ response were stimulated at the lowest concentration (1 μg/ml) of antibody that was tested. Interestingly, anti-μ-Dex at 10 μg/ml, a concentration that did not stimulate [^{3}H] dThd incorporation above that of control, resulted in the highest increase in $[Ca^{2+}]_i$. In addition, at 0.01 μg/ml, anti-μ-Dex stimulated increased B cell proliferation but its effect on $[Ca^{2+}]_i$ was smaller.

3.4 Low concentrations of anti-μ-Dex induce B cell activation but do not rapidly modulate sIgM

Soluble unconjugated anti-Ig antibody is mitogenic at high molar concentrations that result in the rapid modulation of sIg. In contrast, it has been demonstrated that low concentrations of anti-μ-Dex which are mitogenic do not modulate sIg [14]. To study if the situation was similar in human B cells, we studied the ability of anti-μ and anti-μ-Dex antibody to bind to human B cells and modulate sIg. Anti-μ-Dex antibody at 10 μg/ml bound to B cells as well as 10 μg/ml of unconjugated anti-μ as reflected by the equivalent fluorescence seen after addition of FITC-labeled rabbit anti-mouse Ig. However, the binding of 1 μg/ml anti-μ-Dex was very low (Fig. 7a). Modulation of sIg was measured with a fluorescence-labeled polyclonal goat anti-IgM to stain for residual sIgM that remained on anti-IgM-coated B cells that had been cultured for 2 h under capping conditions. Binding of this polyclonal FITC-anti-μ antibody was not inhibited to any detectable degree by a mouse anti-human IgM mAb that had been previously bound to the cells (data not shown). Thus, any diminution in binding of this antibody reflected a decrease in the availability of sIgM as a consequence of modulation of sIg. Unconjugated anti-μ at 10 μg/ml and 1 μg/ml and anti-μ-Dex at 10 μg/ml induced modulation of sIgM, while anti-μ-Dex at 1.0 μg/ml or 0.1 μg/ml (Fig. 7b) did not induce detectable modulation at 2 h. These data indicate that low concentrations of Dex-conjugated antibody that lead to the highest increase in thymidine incorporation do not induce measurable modulation of sIgM within a short time period after binding.

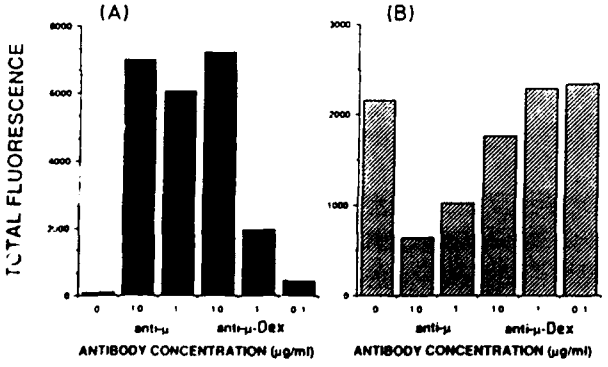

Figure 7. Low doses of anti-μ-Dex do not modulate sIgM. Purified human B cells were incubated with anti-μ or anti-μ-Dex antibodies for 120 min at 4 °C. Separate aliquots of cells were either suspended in medium containing 0.2% sodium azide at 4 °C to prevent sIg modulation (A) or in RPMI 1640 at 37 °C for 2 h to allow sIg modulation (B). Cells were then stained with FITC-conjugated GaMIg (A) or GaHμ (B) antibodies and were analyzed by FCM. Control cells were incubated with medium alone and were stained with FITC-GaMIg.

4 Discussion

Our data demonstrate that anti-μ-Dex is a potent activator of human B lymphocytes when used at ng/ml concentrations. Anti-μ-Dex stimulated large increases in cell size and in $[Ca^{2+}]_i$, and induced B cell proliferation at concentrations which were 1000-fold lower than that required for stimulation by unconjugated anti-μ. The enhanced stimulatory capacity of anti-μ-Dex does not merely reflect its ability to stimulate greater and more prolonged increases in $[Ca^{2+}]_i$ than unconjugated anti-μ since at its most stimulatory concentration it induced lesser increases in $[Ca^{2+}]_i$ than did unconjugated anti-μ. Furthermore, the highest concentrations which stimulated the greatest increase in $[Ca^{2+}]_i$ also stimulated the lowest level of proliferation. It is also unlikely that the carrier molecule, Dex, contributed an ancillary signal which synergized with anti-μ in inducing B cell activation, since co-culture of anti-μ and Dex over a wide range of concentrations gave no enhanced proliferative response. The most likely explanation which can account for the enhanced stimulatory ability of the anti-μ-Dex conjugate is its presentation of a multivalent array of anti-μ molecules on a large carrier molecule. Because of its enhanced cross-linking abilities, considerably lower concentrations which are unable to modulate sIg can be used and thus B cells continue to express sIg. The continued presence of sIg on these B cells allows for continuous and repetitive signaling by these Dex conjugates. This is unlike the situation that is seen with unconjugated anti-Ig were high concentrations of agonist are required to achieve a threshold number of cross-linked sIg molecules to mediate B cell activation. At these concentrations most of the sIg is effectively cleared and thus persistent and prolonged signaling by anti-Ig antibody is minimized. It is likely that this property also accounts for the enhanced stimulatory capacity of the TI type 2 polysaccharide antigens. While they may not attain high concentrations under *in vivo* conditions, their multivalency and large size enables them to cross-link multiple sIg molecules in the absence of detectable modulation and thereby effectively transduce repetitive and prolonged signaling.

At 10 μg/ml, anti-μ-Dex did not induce proliferation but did stimulate the early activational event of increases in $[Ca^{2+}]_i$ and later entry into G_1 (as reflected by cell size increase). These high concentrations of anti-μ-Dex inhibited B cell proliferation that was stimulated by SAC. This suggests that the absence of B cell proliferation at these high concentrations of anti-μ-Dex most likely reflects its ability to inhibit progression of B cells into S rather than its inability to stimulate an early activational signal. The mechanisms of this inhibition are unknown but there are a number of possibilities that we are currently investigating: (a) large and prolonged increases in $[Ca^{2+}]_i$ may mediate a tolerogenic signal as has been demonstrated to occur with T cells [27]; (b) inhibitory lymphokines may be stimulated by "super activated" B cells; and (c) cross-linking of sIg

molecules above a critical threshold number may mediate inhibition of the later but not earlier stages of B cell activation. Although it is not clear which, if any, of these possibilities account for the inhibition of B cell activation, it appears that the absolute magnitude of increases in $[Ca^{2+}]_i$ or in cell size does not bear a direct relationship to the ultimate stimulation of B cell DNA synthesis.

The small amount of increases in detectable $[Ca^{2+}]_i$ elevations by low mitogenic concentrations of anti-μ-Dex support the report of Hivroz et al. [28] who demonstrated that human CLL cells can proliferate in response to anti-μ in the absence of elevations in $[Ca^{2+}]_i$. It also supports our previous work that suggests that other phosphatidylinositol bisphosphate-independent pathways may be recruited in B cells stimulated by anti-Ig [29].

These experiments suggest that anti-μ-Dex may provide a useful model for studying T cell-independent B lymphocyte activation by very low concentrations of agonist that may mimic concentrations achieved by antigens *in vivo*. The fact that these conjugates are soluble and their concentration clearly definable provide additional advantages to that of anti-Ig-Sepharose beads.

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